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translator to RWS Group Ltd, of Europa House, Marsham Way, Gerrards Cross, Buckinghamshire, England declare;

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2. That I am well acquainted with the French and English languages.

3. That the attached is, to the best of my knowledge and belief, a true translation into the English language of the accompanying copy of the specification filed with the application for a patent in France on 8 June 1999 under the number 99/07,457 and the official certificate attached hereto.

4. That I believe that all statements made herein of my own knowledge are true and that all statements made on information and belief are true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the patent application in the United States of America or any patent issuing thereon.

B. Parrish

For and on behalf of RWS Group Ltd

The 6th day of December 2005

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Drawn up in Paris, 08 NOV. 2001

On behalf of the Director-General of the
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The Patent Department Head

(signature)
Martine PLANCHE

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PATENT, UTILITY CERTIFICATE

Intellectual Property Code - Book VI

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REQUEST FOR GRANT

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Reserved for the INPI	
DATE OF SUBMISSION OF THE DOCUMENTS	-8 JUNE 1999
NATIONAL REGISTRATION	99/07,457
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DATE OF FILING	08 JUNE 1999

1. NAME AND ADDRESS OF THE APPLICANT OR THE REPRESENTATIVE TO WHOM THE CORRESPONDENCE IS TO BE ADDRESSED

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PG 04853	PM9905	04.37.37.70.90

2. APPLICATION — Nature of the industrial property right

☒ patent ☐ divisional application → initial application ↓

☐ utility certificate ☐ conversion of a European patent application ☐ patent ☐ utility certificate No date

Compilation of the search report ☐ deferred ☒ immediate

The applicant, as a physical person, asks to pay the fee by instalments ☐ yes ☐ no

Title of the invention (maximum 200 characters)

IMMUNOSTIMULATING OLIGONUCLEOTIDE

3 APPLICANT(S) SIREN No. 3 4 9 5 0 5 3 7 0 APE-NAF code 2 4 4 C

Name and forenames (underline the surname) or company name

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Legal form

S.A.

Nationality/Nationalities FRENCH

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If insufficient space, continue on plain paper ☐

4. INVENTOR(S) The inventors are the applicants ☐ yes ☒ no If the answer is no, provide a separate designation

5. REDUCTION OF THE RATE OF FEES ☐ requested for the first time ☐ requested prior to filing; attach copy of the favourable decision

6. PRIORITY DECLARATION OR APPLICATION FOR THE BENEFIT OF THE FILING DATE OF A PRIOR APPLICATION

Country of origin	Number	Filing date	Nature of the application

7. DIVISIONS previous to the present application — No. date No. date

8. SIGNATURE OF THE APPLICANT OR REPRESENTATIVE (name and capacity of the signatory - registration No.)

(signature)
KERNEIS Danièle
Intellectual Property Engineer

SIGNATURE OF THE RECEIVING OFFICIAL

M. DUEZ

SIGNATURE AFTER REGISTRATION OF THE APPLICATION AT THE INPI

(illegible signature)

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PATENT, UTILITY CERTIFICATE

DESIGNATION OF THE INVENTOR
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PATENTS ADMINISTRATIVE DIVISION

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NATIONAL REGISTRATION NO.

99/07,457

TITLE OF THE INVENTION:

IMMUNOSTIMULATING OLIGONUCLEOTIDE

THE UNDERSIGNED

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NOTE: In exceptional cases, the name of the inventor may be followed by that of the company to which he belongs (membership company) when the latter is other than the company which is the applicant or proprietor.

Date and signature(s) of the applicant(s) or of the representative

8 June 1999
(signature)
KERNEIS Danièle

IMMUNOSTIMULATING OLIGONUCLEOTIDE

The present invention relates to the field of immunostimulants. More particularly, the invention relates to oligonucleotides capable of stimulating human cells which are involved in the immune system, and to their use as a vaccine adjuvant.

A large number of oligonucleotides have already been described in the prior art, in relation to their immunostimulating properties. Thus, application EP 0 468 520 describes immunostimulating polynucleotides consisting of a single strand of linear DNA comprising from 10 to 100 nucleotides which are linked together according to a palindromic sequence.

According to application WO 96/02 555, the immunostimulatory activity of oligonucleotides is linked to the presence of a 5' CG 3' dinucleotide sequence in which neither C nor G are methylated, the immunostimulating activity being stronger if the CG motif is preceded on the 5' side by the GA dinucleotide and/or followed on 3' side by the TC or TT dinucleotide.

On the other hand, according to patent application WO 98/52 962, it is not necessary for the sequence of the oligonucleotide to be a palindrome, or for it to comprise the CG dinucleotide; specifically, the 3 nucleotides described in that application for use as a vaccine adjuvant have the following sequences:
5' GACGTT 3',
5' GAGCTT 3' and
5' TCCGGA 3'.

According to US patent 5 663 153, the immunostimulating activity of oligonucleotides is not linked to the sequence of the nucleotides, but to the nature of the bonding between nucleotides, the presence of at least one phosphorothioate bond making it possible to induce stimulation of the immune system.

Most assays of the prior art for evaluating the immunostimulating activity of the oligonucleotides

proposed, are carried out either in vitro on animal cells (murine cells essentially), or in vivo on mice. However, the differences which exist between the immune system of mice and that of human beings have led to differences between the results obtained on murine cells and those obtained on human cells.

Now, the pharmaceutical industry has a great need for immunostimulants which can be administered to humans, in particular in the vaccine field.

The aim of the present invention is therefore to provide oligonucleotides capable of stimulating human immune system cells.

To achieve this aim, a subject of the invention is an oligonucleotide capable of stimulating human immune system cells, characterized in that it comprises at least one 5' T T N_1 N_2 T T 3' sequence in which T is thymine, and N_1 and N_2 may each represent adenine, thymine, cytosine or guanine, and in that it lacks a CG dinucleotide in which the cytosine C is not methylated.

According to one characteristic of the invention, the oligonucleotide comprises from 6 to 100 nucleotides.

According to another characteristic, the oligonucleotide according to the invention is capable of inducing the proliferation of human lymphocytes.

According to another characteristic, the oligonucleotide according to the invention is capable of increasing the expression of CD25 and CD86 activation markers of human B lymphocytes.

A subject of the invention is also a vaccine adjuvant, characterized in that it comprises at least one oligonucleotide capable of stimulating human immune system cells, which has at least one 5' T T N_1 N_2 T T 3' sequence in which T is thymine, and N_1 and N_2 may each represent adenine, thymine, cytosine or guanine, the oligonucleotide lacking a CG dinucleotide sequence in which the cytosine C is not methylated.

A subject of the invention is also a vaccine composition for human use comprising at least one

vaccine antigen, characterized in that it also comprises at least one oligonucleotide capable of stimulating human immune system cells, which has at least one 5' T T N_1 N_2 T T 3' sequence in which T is thymine, and N_1 and N_2 may each represent adenine, thymine, cytosine or guanine, the oligonucleotide lacking a CG dinucleotide sequence in which the cytosine C is not methylated.

The present invention will be more clearly understood upon reading the description which follows, with reference to figures 1 to 6 which illustrate the results obtained in the assays described in examples 2 and 3.

For the purposes of the present invention, the term "oligonucleotide" is intended to mean a polynucleotide comprising at least 6 nucleotides. In fact, contrary to the teaching of the article entitled "CpG motifs in bacterial DNA trigger direct B-cell activation", Krieg et al., Nature 1995, it has been noted that it is not necessary for the oligonucleotide to have at least 8 nucleotides. However, the upper limit for the size of the oligonucleotides is not really determined. It may, however, be noted that the longer the oligonucleotide, the more difficult it will be to carry out its purification during synthesis steps, and the higher the cost price thereof will be. Furthermore, it is probable that an oligonucleotide of great length will have more difficulty in penetrating into the cells. Thus, for the needs of the present invention, it is considered that a 100-nucleotides size limit for the oligonucleotide is suitable. This oligonuleotide is preferably a single-stranded oligonucleotide; it may be an oligodeoxyribonucleotide or an oligoribonucleotide. Particularly good results have been obtained using an oligodeoxyribonucleotide. The oligonucleotides suitable for the purposes of the invention can be in the form of a phosphodiester or, in order to be more stable, in the form of phosphorothioates or of phosphodiester/phosphorothioate

hybrids. Those preferred are the phosphorothioate oligonucleotides.

The oligonucleotide according to the invention is capable of stimulating human immune system cells. This stimulation is distinguished in particular by lymphoproliferation or by the expression of activation markers, such as CD25 and CD86 markers of B lymphocytes. It is possible to select oligonucleotides of interest using assays other than those provided in the present application, on condition, however, that they are assays which evaluate the capacity for stimulating human cells and not, as in most documents of the prior art, assays which evaluate the capacity for stimulating murine cells. It would in particular be possible to assay the expression of other B lymphocyte activation markers such as CD69 or CD56, or the expression of proliferation markers such as the KI67 marker; assays relating to the increase in activation and maturation markers for dendritic cells might also be used. Similarly, assays enabling the assessment of the increase in production of certain cytokines such as, for example, IL6, IL12, IL10, IFNγ, and TNFα may also be used.

According to one characteristic of the invention, the oligonucleotide comprises at least one 5' T T N_1 N_2 T T 3' nucleotide sequence in which T means thymine, and N_1 and N_2 may each represent adenine, thymine, cytosine or guanine. This formula therefore includes 16 possibilities. This sequence can be a 5' terminal sequence or a 3' terminal sequence, or be surrounded by other nucleotides. It can be unique or repeated identically several times within the same oligonucleotide. An oligonucleotide according to the invention may also comprise several different sequences, each corresponding to the 5' T T N_1 N_2 T T 3' motif.

According to the invention, the oligonucleotide does not include a palindromic sequence. Despite this absence of palindromic sequence, such an

oligonucleotide is capable of stimulating human immune system cells.

According to one characteristic, the oligonucleotide according to the invention lacks a CG dinucleotide in which the cytosine is not methylated. This exclusion also applies to the N_1 N_2 motif. The capacity of the oligonucleotides of the prior art to be immunostimulating has almost always been interpreted as being linked to the presence of nonmethylated CpG motifs (Cf. in particular the article by Krieg et al in Nature of April 1995, mentioned above), this interpretation being coherent with the observation according to which the frequency of this dinucleotide is 4 times greater in the genome of bacteria and viruses than in that of vertebrates. Surprisingly, it has now been found that oligonucleotides which entirely lack this dinucleotide motif are, however, perfectly capable of stimulating the human immune system.

According to one particular characteristic, the oligonucleotide according to the invention lacks or is low in nucleotide sequence capable of inhibiting human immune system cells. Specifically, in order to obtain an overall immunostimulating effect, if inhibitory or neutralizing motifs such as, for example, those described in application WO 98/52 581 are present, their effect must be suppressed or reduced, through the presence of a sequence with a more pronounced immunostimulating effect, or through the presence of a greater number of 5' T T N_1 N_2 T T 3' sequences.

A subject of the present invention is also a vaccine adjuvant comprising at least one immunostimulating oligonucleotide which has at least one 5' T T N_1 N_2 T T 3' motif as mentioned above. The term "vaccine adjuvant" is intended to mean a product which makes it possible to increase or to modify the response of an organism's immune system with respect to the administration of an antigen. In particular, it may be an increase in the humoral response or in the cellular response.

The action of a vaccine adjuvant can also be, not an increase in the response which would occur in the absence of an adjuvant, but a different direction of the response produced; for example, directed toward a cellular response rather than a humoral response, production of certain cytokines rather than of others, production of certain types or subtypes of antibody rather than of others, stimulation of certain cells rather than of others, etc.

The immunostimulating oligonucleotide of the present invention can be used as a vaccine adjuvant whatever the nature of the antigen administered and whatever the number of valences used. It may be the only adjuvant used or, conversely, be one element of an adjuvant combination.

The adjuvant action of the oligonucleotide according to the invention can be obtained either when it is combined with the antigen, or with antigens, at the time of their administration, i.e. when they form part of the same vaccine composition, or when it is administered separately from the antigen, or from antigens. It is preferred, however, to use it in the same vaccine composition as the antigen or antigens to be administered.

The oligonucleotide according to the invention can advantageously be administered via all the routes likely to be used for a vaccine composition: mucous membrane route or systemic route.

One of the subjects of the invention is a vaccine composition comprising at least one immunostimulating oligonucleotide which has a 5' T T N_1 N_2 T T 3' sequence as described above.

A vaccine composition according to the invention can be intended for immunization against a single disease, or intended for immunization against several diseases. It can be a liquid or lyophilized vaccine composition. It can comprise, besides the antigens, all or part of the components conventionally present in a vaccine: buffers, stabilizers,

preservatives, etc. It can also comprise one or more adjuvant(s) other than those which are the subjects of the present invention. It can also comprise several adjuvants which are subjects of the present invention, consisting either of oligonucleotides which all have the same 5' T T N_1 N_2 T T 3' motif but which differ by the nucleotides in the 5' and/or 3' regions, or of oligonucleotides which have different 5' T T N_1 N_2 T T 3' motifs, in which the sequences in the 5' region and in the 3' region are identical or different.

The vaccine composition according to the invention can be intended for prophylactic administration or for therapeutic administration.

The vaccine composition according to the invention can be formulated so as to optimize the adjuvant action of the oligonucleotide which is a subject of the invention. Thus, the oligonucleotide can be coupled to a lipid, such as cholesterol; it can be integrated into an emulsion of oil/water type or formulated in the form of liposomes.

The examples which follow illustrate particular embodiments of the present invention.

Example 1:

Oligonucleotide synthesis

15 oligonucleotides are synthesized, each having one of the following motifs:

5' T T A A T T 3'
5' T T A C T T 3'
5' T T A T T T 3'
5' T T A G T T 3'

} A series

5' T T T T T T 3'
5' T T T A T T 3'
5' T T T C T T 3'
5' T T T G T T 3'
} T series

5' T T C C T T 3'
5' T T C A T T 3'
5' T T C T T T 3'
} C series

5' T T G G T T 3'
5' T T G A T T 3'
5' T T G T T T 3'
5' T T G C T T 3'
} G series

And having 4 adénines in the 5′ position and 5 adenins in the 3′ position.

The synthesis of these oligonucleotides is performed by means of a synthesizer machine supplied by Applied Biosystems, which uses the standard phosphoramidite chemical method and which, in each cycle, includes an oxidation step which is carried out by means of a tetraethylthiuram/acetonitrile solution so as to obtain a phosphorothioate bond.

In the same way, an oligonucleotide A15(S) is also prepared which comprises only As and which is phosphorothioate along its entire length. This oligonucleotide is a negative control since it causes neither proliferation of nor an increase in the expression of activation markers of B lymphocytes.

An oligonucleotide 3Db(S) is also prepared, the sequence of which is described in patent application WO 96/02555 under SEQ ID No. 15 (5'GAGAACGCTCGACCTTCGAT3'); this oligonucleotide comprises phosphorothioate bonds along its entire length and is used as a positive control.

All the oligonucleotides are maintained in solution in PBS buffer.

Example 2:

Lymphoproliferation Assay

Lymphocytes are isolated from the peripheral blood of a donor by carrying out a centrifugation on a Ficoll gradient. These lymphocytes are adjusted to 2.10^6 cells/ml in culture medium (RPMI 1640 + 10% of foetal calf serum as well as glutamine, streptomycin and penicillin).

The cells are distributed into 96-well plates (round-bottomed) in 100 µl, i.e. 2.10^5 cells per well. 100 µl of a 4 µM solution of oligonucleotides to be assayed produced in example 1 (only 1 oligonucleotide type per well) are added so as to obtain a 2 µM final concentration.

The cells are incubated for 48 to 72 hours.

Tritiated thymidine (Amersham TRK 120) is diluted in culture medium and then distributed in the plates in a proportion of 1 µCi per well in 50 µl. After 7 to 8 hours of incubation in an incubator (5% CO_2, 37°C), the plates can be frozen at -80°C and processed later.

Using the "Harvester", the content of the wells is harvested on Unifilter GF/C plates, and 6 washes in distilled water and then one wash in 70% ethanol, in order to precipitate the DNA, are carried out.

After drying the plates, 25 µl of scintillation liquid (Microscint-40, Packard) are distributed into each well and make it possible to quantify the radioactivity (radiation emitted by the tritium) by measuring the number of counts/minute (cpm) emitted by each well, in a Top Count counter (Packard).

The results obtained for each of the oligonucleotides assayed are given in figures 1 and 2, which indicate the number of counts per minute for each oligonucleotide tested; it is observed that all the

oligonucleotides according to the invention give a result which is distinctly better that the result obtained with the medium on its own or the negative control A15(S), which means that they are all capable of stimulating the proliferation of lymphocytes.

Example 3:

Assay relating to activation markers

The assay is carried out using lymphocytes isolated from a donor as described in the preceding example and adjusted to 2.10^6 cells/ml in the same culture medium.

The cells are then distributed in 12-well plates in a volume of 2 ml, i.e. 4.10^6 cells/well. An amount of oligonucleotides to be tested prepared in example 1 (1 oligonucleotide/well) sufficient to obtain a 2 μM oligonucleotide concentration is added to each well. The cells are then incubated for 72 hours at 37°C. The cells are then double-labelled using CD25PE/CD20FITC or CD86PE/CD20FITC, and then analysed on FACScan. The results obtained are illustrated on figures 3, 4, 5 and 6, which represent, for each oligonucleotide tested, the percentage of B cells (CD20+) which express the CD25 marker (those which are CD25+) or the CD86 marker (those which are CD86+). The results represented on figures 3 and 4 were obtained in an assay carried out at a time which was different from the assay of which the results are illustrated on figures 5 and 6, which explains the order of magnitude difference in the number of counts per minute measured. In fact, in this type of manipulation, the assays are very variable from one assay to the other, and only different results obtained in the same assay can be compared with one another, hence the need to include, in each assay, a control oligonucleotide and also an assay of the medium alone.

It is noted that all the oligonucleotides which are the subject of the invention activate B lymphocytes which express their activation marker CD25 and CD86.

Claims

1. Oligonucleotide capable of stimulating human immune system cells, characterized in that it comprises at least one nucleotide sequence which has the following formula 5' T T N_1 N_2 T T 3', in which T means thymine, N_1 and N_2 may each represent adenine, thymine, cytosine or guanine, and in that it lacks a CG dinucleotide in which the cytosine C is not methylated.

2. Oligonucleotide according to claim 1, characterized in that it comprises from 6 to 100 nucleotides.

3. Oligonucleotide according to one of the preceding claims, characterized in that it is capable of inducing the proliferation of human B lymphocytes.

4. Oligonucleotide according to one of the preceding claims, characterized in that it is capable of increasing the expression of the CD25 and CD86 activation markers of human B lymphocytes.

5. Vaccine adjuvant, characterized in that it comprises at least one oligonucleotide according to one of claims 1 to 4.

6. Vaccine composition for human use comprising at least one vaccine antigen, characterized in that it also comprises at least one oligonucleotide according to one of claims 1 to 4.

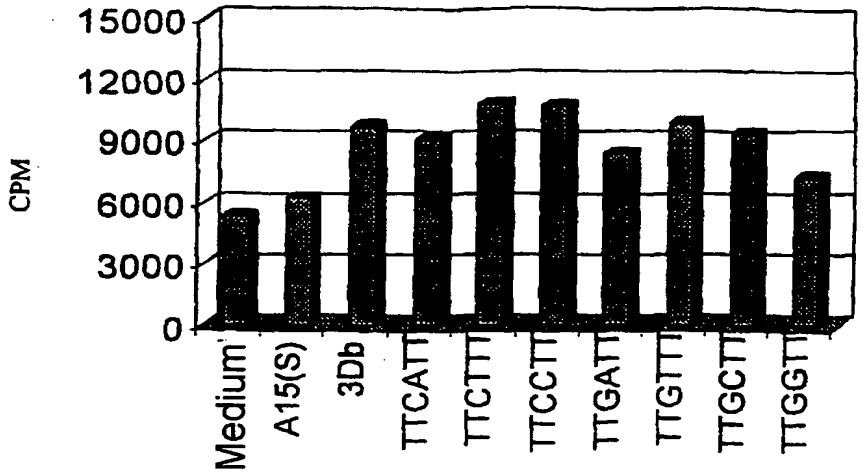

Figure 1

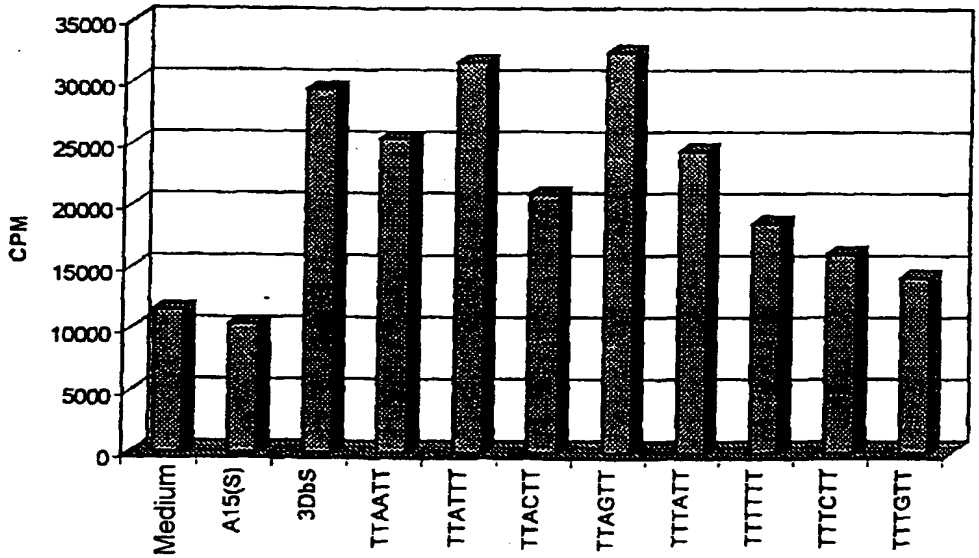

Figure 2

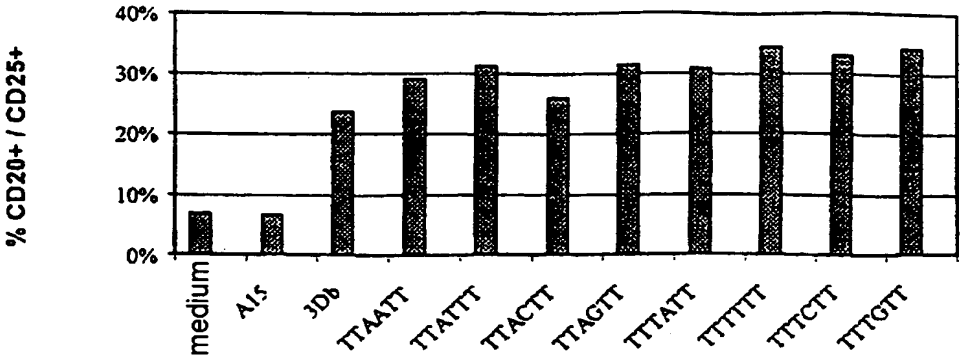

Figure 3

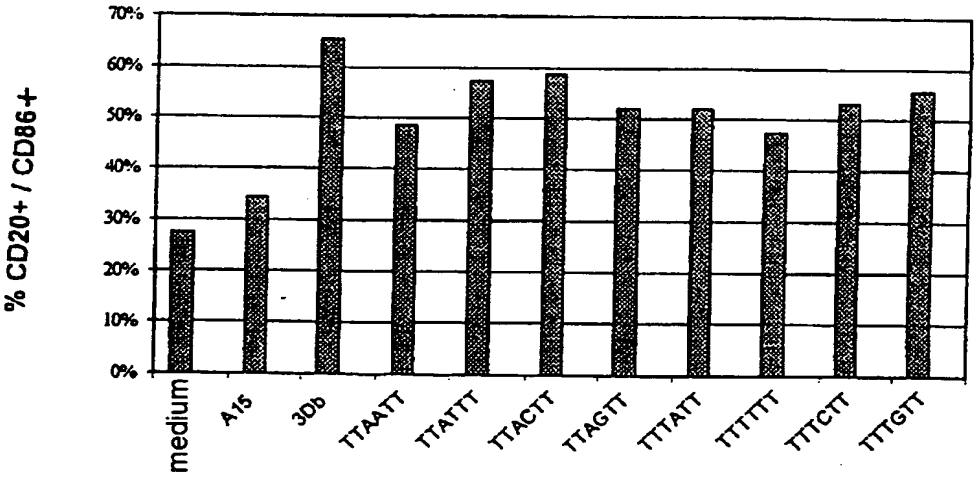

Figure 4

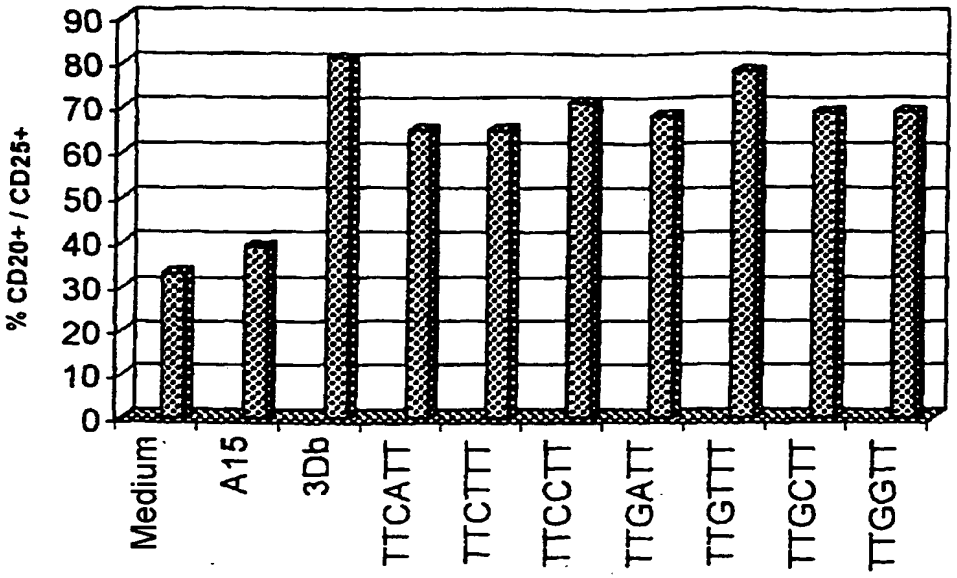

Figure 5

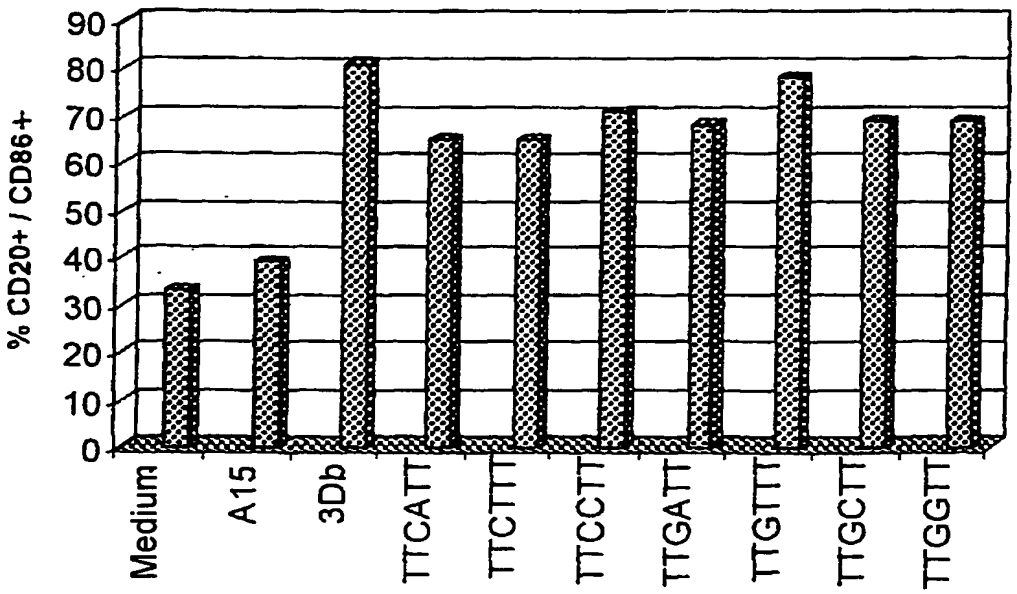

Figure 6